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First report of *Penicillium brasilianum* Bat., *P. cluniae* Quintan., and *P. echinulonalgiovense* S. Abe ex Houbraken & R.N. Barbosa (Eurotiales, Aspergillaceae) as endophytes from a bromeliad in the Caatinga dry forest in Brazil

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Abstract

Penicillium brasilianum Bat., *P. cluniae* Quintan., and *P. echinulonalgiovense* S. Abe ex Houbraken & R.N. Barbosa are reported for the first time as endophytes from the leaves of an endemic bromeliad in the Caatinga dry forest in Brazil. For species determination, phenotypic features were analysed along with the sequencing of the β-tubulin and calmodulin genes. *Penicillium* Link isolates obtained in this study showed the typical morphology of species in the *Lanata-Divaricata* section. These results contributed to increase the knowledge of fungal diversity in dry environments in the world.

Keywords

Aspergillaceae, biodiversity, Brazilian fungi, endophytic fungi, dry ecosystems.

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Introduction

Penicillium species are found on a wide variety of substrates and are the most well distributed fungi in the world (Visagie et al. 2014). These fungi are of great importance in the fields of medicine, agriculture, production of industrially important enzymes, decomposition of organic matter such as food, and indoor environments (Houbraken et al. 2014). They are also often referred to as endophytes in several ecosystems, including tropical dry forests (e.g., Bezerra et al. 2015; Silva et al. 2018; Pádua et al. 2019).

In Brazil, one of the tropical dry forests, named Caatinga, primarily occupies the northeast region of the country (MMA 2013). The Caatinga dry forest is enriched with Bromeliaceae, Cactaceae, and Euphorbiaceae species (Pontes and Agra 2006). Among the species of bromeliads, *Tillandsia catimbauensis* Leme, W. Till & J.A. Siqueira is an endemic plant in Brazil, native to the Caatinga region, and is currently at critical risk of extinction (Forzza et al. 2015). These bromeliads species are found in one of the largest Caatinga protected

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area, the Catimbau National Park, which protects about 62,294 hectares of the Caatinga forest (ICMBio 2018). Several studies have already been carried out in the Catimbau National Park with the goals of investigating its fungal diversity (Oliveira et al. 2013; Maia 2014; Barbosa et al. 2016; Lima et al. 2016); however, fewer studies have been conducted to verify the association of endophytic fungi with the Caatinga plants.

Some studies have verified the richness of endophytes associated with bromeliads in Brazil (e.g., Landell et al. 2006; Felix et al. 2017; Bezerra et al. 2018); however, until now, only one study validated the presence of endophytic fungi associated with bromeliads in the Caatinga forest (Silva et al. 2018), but without a full identification of strains at the species level.

The aim of the present study was to identify endophytic *Penicillium* isolates from Silva et al. (2018), belonging to the section *Lanata-Divaricata*, by combining morphological data and sequences of two genes, β-tubulin (*BenA*) and calmodulin (*CaM*). In this paper, we highlight the occurrence *Penicillium brasilianum* Batista, *P. cluniae* Quintanilla, and *P. echinulonalgiovense* S. Abe ex Houbraken & R.N. Barbosa for the first time as endophytes in the Caatinga dry forest in Brazil.

Methods

Endophytic fungi. The endophytes used in this study were isolated by Silva et al. (2018) from the leaves of *Tillandsia catimbauensis*, a native species in the Brazilian tropical dry forest (Caatinga), Catimbau National Park, Buíque municipality, Pernambuco state, Brazil (08°36′35″S, 037°14′40″W) (Fig. 1). The collections were authorized by the Ministério do Meio Ambiente (MMA)/ Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio); permission number: 48641-1/authentication code 17827693 issued on April 10, 2015.

Morphological analyses. For the analysis of the macroscopic morphological characteristics, 1 µL of the spore suspension was inoculated at three points in each Petri plate containing one of the following culture media: Czapek yeast extract agar (CYA), malt extract agar (MEA), oatmeal agar (OA), dichloran glycerol agar (DG18), Czapek sucrose extract agar (CYAS), yeast extract sucrose agar (YES), and creatine sucrose agar (CREA). Plates were incubated at 15 °C and 25 °C for seven days, and only the plates containing the CYA and MEA were further incubated at 30 °C and 37 °C for seven days. For the macro morphological observations, the fungi were grown at 25 °C (Houbraken et al. 2011). After the incubation period, macroscopic analysis of the cultures was performed, and lactic acid slides were prepared from the colonies grown in MEA for microscopic analysis. Colony colours were analysed according to the colour charts of Rayner (1970). Following recommendations by Barbosa et al. (2020), representative isolates are deposited in the culture collection Micoteca URM Profa. Maria Auxiliadora Cavalcanti (WCDM 604), at the Federal University of Pernambuco, Recife, Brazil.

DNA extraction, PCR, and sequences analyses. Genomic DNA was extracted from the biomass of the *Penicillium* cultures grown on MEA after seven days at 25 °C using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The ITS rDNA region, *BenA* gene, *CaM* gene, and the major subunit of RNA polymerase (*RPB2*) were amplified as described by Visagie et al. (2014). Amplification and sequencing reactions, sequences analyses, and consensus of sequences were performed as described previously by Visagie et al. (2014).

Phylogenetic analyses. β-tubulin and calmodulin sequences were chosen based on published literatures (Barbosa et al. 2018; Diao et al. 2018; Kubátová et al. 2019) and included in the matrix along with the endophytic *Penicillium* sequences of this study. Sequences were aligned using MAFFT v.7 (Katoh and Standley 2013) and manually improved in MEGA v. 7 (Kumar et al.

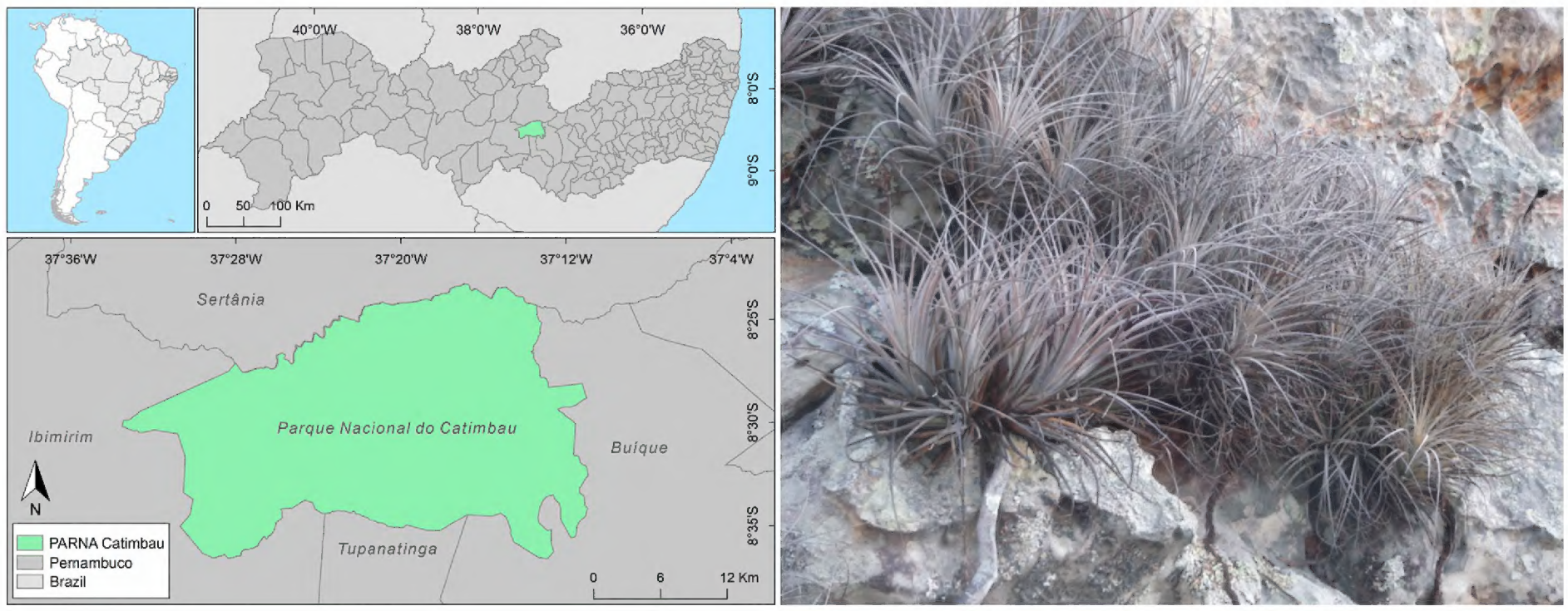

Figure 1. The geographical location of the Catimbau National Park in Brazil (highlighted in green), and the bromeliad *Tillandsia catimbauensis*.

2016). The maximum likelihood (ML) analysis was performed in RAxML-HPC v. 8.2.8 Black Box (Stamatakis 2014) using the GTR + G + I model test. The Bayesian inference (BI) was performed in MrBayes v. 3.2.2 (Ronquist et al. 2012) on XSEDE using the best nucleotide model (β-tubulin = K80+G, and calmodulin = TrN+G) as estimated using the MrModelTest v.2.3 (Nylander 2004). BI analysis was conducted with 1×10^6 generations, a burning value of 25% and chains were sampled every 1,000 generations. Both analyses were conducted at the CIPRES Science Gateway (Miller et al. 2010). Phylogenetic trees were viewed and arranged using FigTree v. 1.1.2 (Rambaut 2009). The newly obtained sequences are deposited in the National Center for Biotechnology Information (NCBI; Supplemental Data, Table S1).

Results

***Penicillium brasilianum* Batista, 1957**; Anais da Sociedade de Biologia de Pernambuco 15 (1): 160. Figures 2 A–D

Material examined. BRAZIL • Pernambuco, Catimbau National Park; 08°36′35″S, 037°14′40″W; Jun. 2015; K.T.L.S. Freire leg.; isolated as endophytic fungus from *Tillandsia catimbauensis*. URM 7667, URM 7668, T26.125, T12.129, T98.132, T59.139, T25.154, T143.177, T130.179, and T49.182.

Distribution. Brazil, Canada, Costa Rica, Czechoslovakia, Denmark, Germany, Iraq, Japan, Korea, Netherlands, South Africa, USA, USSR and Zimbabwe.

Identification (colony characters, 25 °C, 7 days). CYA: colonies low, plane, texture velvety, strong sporulation; greyish to turquoise-green in margins, whitish margin, hyaline mycelium, reverse yellowish cream; exudate absent. MEA: colonies texture velvety, greyish-green with whitish margins, hyaline mycelium, reverse yellowish to light brown; exudate absent. YES: colony texture velvety, sulcate, colonies low to moderately deep, centrally dark green, coloration ranging from green to greyish, small white margin, reverse yellow; exudate absent. OA: colony texture velvety, plane, turquoise-green to greyish, whitish margin to yellowish, reverse yellow pigmented; hyaline exudate observed. CYAS: colonies plane, low, texture velvety, turquoise-green for reverse greyish cream with dark margin (dark green) or whitish margin; exudate absent. DG18: colonies velvety, turquoise-green, white-green margin, reverse brownish; exudate absent. CREA: poor to moderate growth, acid production absent.

Colony diameters, 7 days, in mm. CYA 15 °C 22–23; CYA 25 °C 39–40; CYA 30 °C 39–40; CYA 37 °C 10–12; MEA 15 °C 25; MEA 25 °C 54–55; MEA 30 °C 50–51; MEA 37 °C 7–8; YES 25 °C 50; DG18 25 °C 24–26; CYAS 25 °C 29–32; OA 25 °C 42–43; CREA 25 °C 27.

Micromorphology (25 °C, 7 days). MEA: conidiophores long, up to 600 µm in length, 3–3.5 µm wide, biverticillate, frequently with lateral branches; stipes/metulae rough walled, 10–14 × 3.0 µm, stipe with three metulae, with spatulate termination, with 3–4(–5) phialides per stipe; phialides ampulliform 7.5–10.5 × 3.0 µm; branches 11.5–21 × 3 µm, metulae 7.5–19 × 3 µm, phialides 7.5–13 × 3 µm; conidia finely rough walled to rarely echinulate, subglobose to ellipsoidal, conidia without pigmentation, 2–4 × 2–3 µm.

Comments. In our phylogenetic analyses endophytic isolates grouped in the same clade, including *P. brasilianum* CBS 253.55 ex-type strains (Fig. 3), and they also had morphological similarities to *P. brasilianum*, which was firstly found on herbarium exsiccata and it is mainly characterized by ampulliform phialides (5–8 per metulae/stipe, 5.5–10 × 2.5 µm), rami/branches (8–20 × 2–3 µm), and by the production of pigmented conidia (3–6 × 4.5–5 µm) (Batista and Maia 1957). Thus, *P. brasilianum* is here reported as endophytic fungus for the first time.

***Penicillium cluniae* Quintanilla, 1990**; Avances en Alimentación y Mejora Animal 30 (4): 174. Figures 2 E–H

Material examined. BRAZIL • Pernambuco, Catimbau National Park; 08°36′35″S, 037°14′40″W; Jun. 2015; K.T.L.S. Freire leg.; isolated as endophytic fungus from *Tillandsia catimbauensis*; URM 7665 and URM 7666.

Distribution. Brazil, Czech Republic, Netherlands, Spain, and USA.

Identification (colony characters, 25 °C, 7 days). CYA: colonies velvety, radially sulcate; centrally grey to turquoise-green, whitish margin, slightly elevated, sparse hyaline exudate, reverse beige. MEA: colonies velvety, greyish-green with slightly greenish margins, strongly sporulated, exudate absent, reverse yellow-brown. YES: colonies velvety, radially sulcate, and slightly concentrically sulcate; greenish-grey with whitish margins; exudate absent; reverse yellow-brown. OA: colonies low, plane, texture velvety, greyish-green with slightly whitish margins; hyaline exudate moderately observed; reverse whitish. CYAS: colonies velvety, greenish-grey coloration with whitish margins; exudate absent; reverse beige-greyish, centrally brownish. DG18: colonies velvety, plane, centrally raised, greyish-green with whitish margins, reverse brownish with clear margins; moderate sporulation; exudate absent. CREA: poor to moderate growth, acid production absent.

Colony diameters, 7 days, in mm. CYA 15 °C 18–19; CYA 25 °C 35–37; CYA 30 °C 39–40; CYA 37 °C 15–20; MEA 15 °C 24–26; MEA 25 °C 47–51; MEA 30 °C 57–58; MEA 37 °C 24–31; YES 25 °C 47–52; DG18 25 °C 24–30; CYAS 25 °C 30–31; OA 25 °C 36–37; CREA 25 °C 24–31.

Micromorphology (25 °C, 7 days). MEA: conidiophores longs, up to 600 µm length, 2.5–3.5 µm wide, monoverticillate, most often with a distinct branch, biverticillate conidiophores rarely observed; stipes/metulae smooth walled, 13–27.5 × 2.5–3.5 µm, regularly septate (3–5

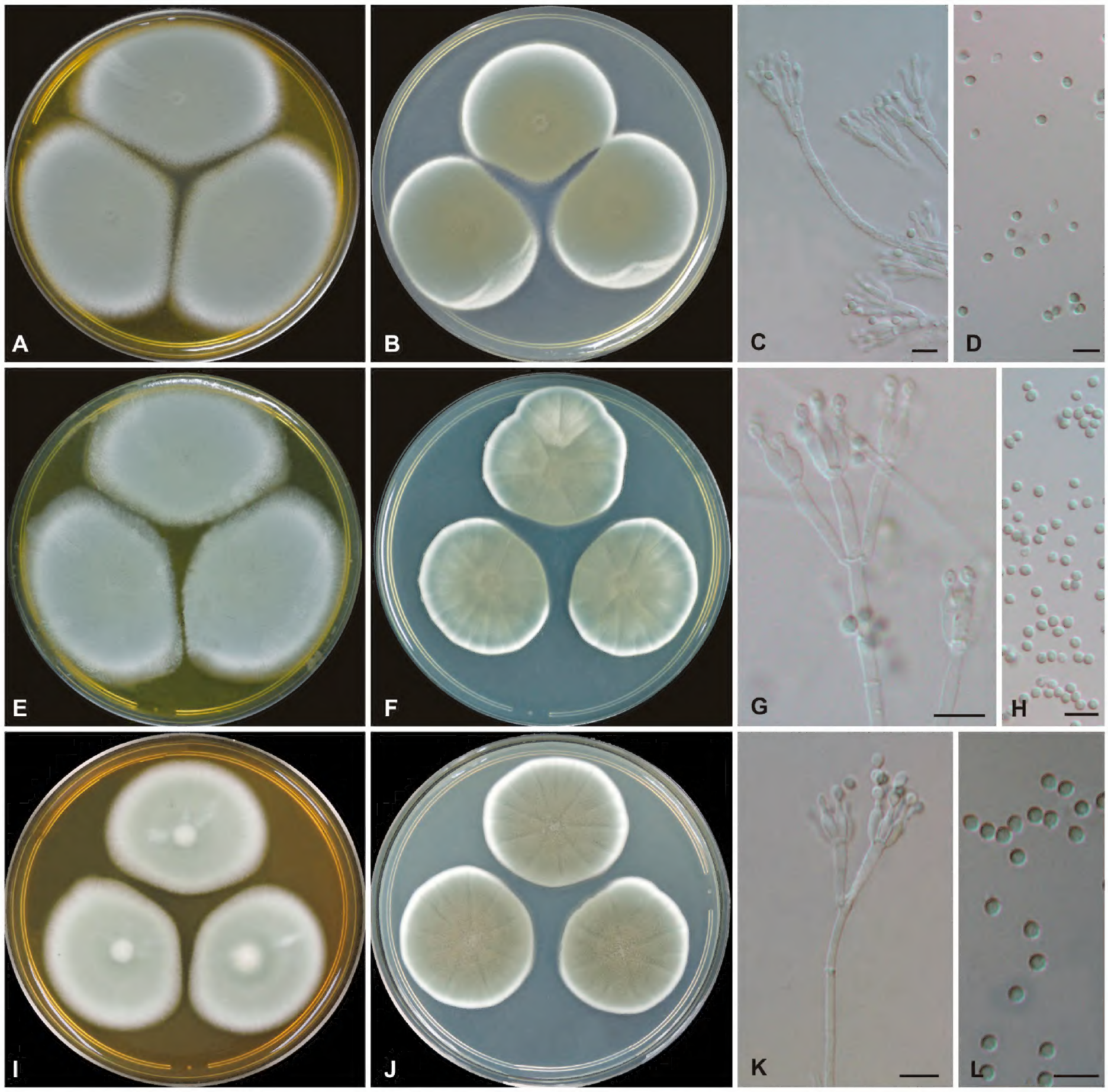

Figure 2. A–D. *Penicillium brasilianum*. **A.** Colonies on MEA. **B.** Colonies on CYA. **C.** Conidiophores and conidia. **D.** Conidia. **E–H.** *Penicillium cluniae*. **E.** Colonies on MEA. **F.** Colonies on CYA. **G.** Conidiophores and conidia. **H.** Conidia. **I–L.** *Penicillium echinulonalgiovense*. **I.** Colonies on MEA. **J.** Colonies on CYA. **K.** Conidiophores and conidia. **L.** Conidia. Scale bars: 10 µm.

septate), with a spatulate termination (2.5–3.5 µm), often with 2–4 phialides, rarely 5 phialides per stipe; phialides ampulliform 8.5–10(–15) × 3–3.5 µm; conidia rough walled, commonly globose to subglobose, rarely ellipsoidal, greenish pigmented, 2.5–3 (–4.5) × 2–2.5 (–3.5) µm.

Comments. The ex-type strain of *P. cluniae* was first isolated from an uncultivated soil in Spain (Quintanilla Sáez 1990). *Penicillium cluniae* was also reported with insecticidal activity (López-Gresa et al. 2006) and producing the enzyme L-asparaginase (Silva et al. 2018). The endophytic isolates obtained in this study are morphologically similar to *P. cluniae* and phylogenetically placed with high support (BPP = 1 and ML-BS = 89) in the same clade of *P. cluniae* CBS 326.89 ex-type strain (Fig. 3). This is the first record of this species as an endophyte.

***Penicillium echinulonalgiovense* S. Abe ex Houbraken & R.N. Barbosa, 2018**; Antonie van Leeuwenhoek 111 (10): 1895.
Figures 2 I–L

Material examined. BRAZIL • Pernambuco, Catimbau National Park; 08°36′35″S, 037°14′40″W; Jun. 2015; K.T.L.S. Freire leg.; isolated as endophytic fungus from *Tillandsia catimbauensis*; URM 7669.

Distribution. Australia, Brazil, China, Indonesia, Japan, Madagascar, Malaysia, Netherlands, and USA.

Identification (colony characters, 25 °C, 7 days). CYA: colonies velvety, radially sulcate; centrally dark grey to turquoise-green, whitish margin, reverse yellowish-cream; exudate absent. MEA: colonies velvety, centrally elevated, greyish-green with whitish margins, reverse yellowish; exudate absent. YES: colonies velvety to

floccose, radially and centrally sulcate; whitish, centrally turquoise-green to greyish with whitish margins, reverse brownish with yellowish margins, radially and centrally sulcate; exudate absent. OA: colonies velvety, plane, greenish to turquoise-green, whitish margins, reverse brownish; exudate absent. CYAS: colonies velvety, greenish-grey coloration with whitish margins; irregularly sulcate; reverse olive; exudate absent. DG18: colonies velvety, plane, greyish-green with whitish margins; reverse dark brown with light brown margins; exudate absent. CREA: poor to moderate growth, acid production absent.

Colony diameters, 7 days, in mm. CYA 25 °C 34–36; CYA 15 °C 22–23; CYA 30 °C 34–39; CYA 37 °C 8–14; MEA 15 °C 25; MEA 25 °C 39–40; MEA 30 °C 27–30; MEA 37 °C 6–8; CREA 25 ° 18-20.

Micromorphology (25 °C, 7 days). MEA: conidiophores 130–260 × 3.5 µm, monoverticillate, most of with a distinct branch, biverticillate and terverticillate conidiophores rarely observed, spatulate termination; stipe/metulae rough walled, 13.5–19 × 2–2.5 µm, often with 3–5(–6) phialides per stipe; phialides ampulliform (7.5–)8.5–9.5(–10.5) × 2–2.5(–3) µm; conidia globose to subglobose, slightly echinulate, pigment absent, 2–3 × 2–2.5 µm.

Comments. *Penicillium echinulonalgiovense* has been reported from an unrecorded source in Japan; soil in Australia, China, Madagascar, Malaysia, and USA; storage room in Indonesia; industrial installation in the Netherlands; and bee pollen and inside of nests of *Melipona scutellaris* Latreille, 1811 in Brazil (Barbosa et al. 2018). Phylogenetic analysis placed the endophyte URM 7669 grouped with *P. echinulonalgiovense* CBS 328.59 ex-type strain as a single lineage (BPP = 0.94 and ML-BS = 98) in the section *Lanata-Divaricata* (Fig. 3). The isolation of *P. echinulonalgiovense* from the leaves of *T. catimbauensis* in Brazil, is the first report of this species as an endophyte, and demonstrates its plasticity to be isolated from different substrates and hosts.

Discussion

The results presented in this study were obtained through the combination of morphological and molecular data, whose phylogenetic analyses indicated the presence of three *Penicillium* species (*P. brasilianum*, *P. cluniae*, and *P. echinulonalgiovense*) in the *Lanata-Divaricata* section, being reported as endophytes for the first time. The *Penicillium* section *Lanata-Divaricata* comprises of around 75 species, and some authors have described

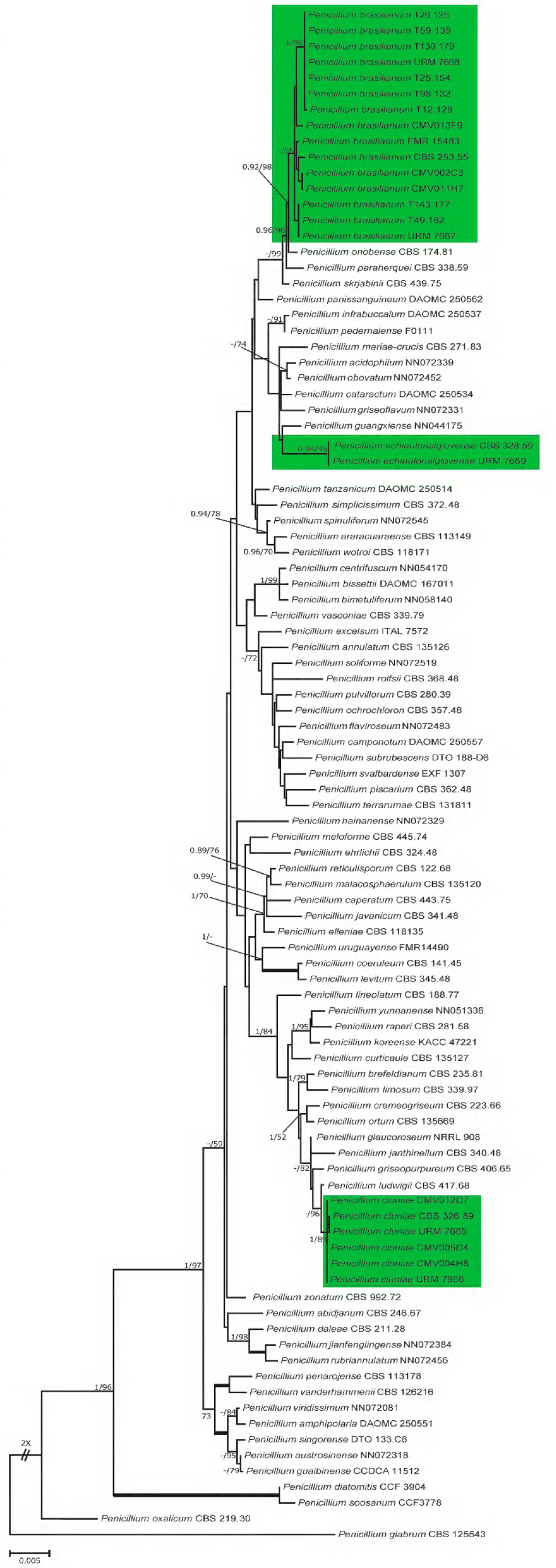

Figure 3. Bayesian inference (BI) tree obtained using a combined *BenA* and *CaM* dataset from species belonging to *Penicillium* section *Lanata-Divaricata*. Species treated in this study are highlighted in green. BPP and ML-BS equal or above 0.89 and 70%, respectively, are shown near nodes. Branches with full support (BPP = 1 and ML-BS = 100%) are thickened. *Penicillium glabrum* CBS 125543 was used as outgroup.

the representatives of this section as divaricate with subterminal to terminal metulae, in intercalary positions (associated with monoverticilate conidiophores), with the members being highly similar to each other and also capable of displaying variations within the same species (Houbraken and Samson 2011; Visagie et al. 2015; Barbosa et al. 2018; Diao et al. 2018).

The endophytic isolates obtained in this study are similar to the description of *Penicillium brasilianum* by Batista and Maia (1957) which used macro and micromorphological features of colonies in Czapek agar medium after 10 days of growth. In another study published by Cho et al. (2005), *P. brasilianum* was reported to be isolated, for the first time, from the soil of a forest in Korea. The authors cultured the colonies in MEA after seven days at 25 ºC. The authors also performed phylogenetic analyses based on sequences of the *BenA* gene and confirmed its sequence identity with the corresponding sequence in other species initially defined by macroscopic and microscopic morphological characteristics.

Based on the phylogenetic analyses and morphological features, two of our endophytes were identified as *P. cluniae*. *Penicillium cluniae* was described by Quintanilla Sáez (1990) and confirmed by Houbraken and Samson (2011) as belonging to the section *Lanata-Divaricata*. Visagie et al. (2015) included *P. cluniae* in a study of the phylogeny of species belonging to this section and described some characteristics of the species, such as absence of acid production in CREA medium and faster growth in CYA (48–50 mm), DG18 (35–38 mm), and CYAS (36–37 mm). In addition, Visagie et al. (2015) demonstrated, based on phylogenetic inferences, the distinction between *P. cluniae* and *P. glaucoroseum*, and reported that these species are closely related to *P. janthinellum* Biourge.

Penicillium echinulonalgiovense was also reported in this study for the first time as an endophyte. The type material of this species was isolated from unrecorded source in Japan (Abe 1956), and it was recently validated by Barbosa et al. (2018) who included it in the *Penicillium* section *Lanata-Divaricata*. Primarily, *P. echinulonalgiovense* has been isolated from soil, storage room, industrial installation, and substrates of *Melipona scutellaris* in Brazil (Barbosa et al. 2018). This study revealed the occurrence of species belonging to *Penicillium* section *Lanata-Divaricata* for the first time as endophytes isolated from the leaves of *Tillandsia catimbauensis*, a native bromeliad of the Caatinga dry tropical forest. This study also demonstrated the richness of *Penicillium* species associated with plants of dry environments, and the importance of preserving these plants in their natural environment.

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Authors' Contributions

The project has been conducted by all authors. KTLSF, GRAM, and RNB carried out the fieldwork. KTLSF, GRAM, and SSN worked with endophytes isolation, purification, and preservation. KTLSF, LMP, RNB, JDPB, CMZM worked during the fungal analyses (morphology and molecular biology) and wrote the manuscript.

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Supplemental Data

Table S1. GenBank accession numbers of sequences from endophytic *Penicillium* isolated from leaves of *Tillandsia catimbauensis* in Brazil included in the phylogenetic analyses. *BenA* sequences obtained from Silva et al. (2018).